Irinotecan-Induced Apoptosis Is Inhibited by Increased P-Glycoprotein Expression and Decreased p53 in Human Hepatocellular Carcinoma Cells

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Irinotecan, a DNA topoisomerase I inhibitor, is widely used in cancer chemotherapy. However, little is known of the mechanisms of its antitumor effects and the development of drug resistance in human hepatocellular carcinoma (HCC). In this study, we investigated the effects of short-term culture with SN-38, the active metabolite of irinotecan, on apoptosis in Huh7 cells. The cells were cultured with SN-38 for 24, 72, and 120 h, and apoptosis was determined using the terminal deoxynucleotidyl transferase (TdT) nick-end labeling (TUNEL) assay. The expressions of p53, apoptosis-related proteins, and P-glycoprotein (P-gp), a protein conferring the multidrug-resistant phenotype, were analyzed using Western blotting. Induced expression of P-gp was detected using fluorescence microscopy. SN-38 significantly induced apoptosis in Huh7 cells at 24 h. SN-38 also increased the expression of p53, Bax, and caspase-9 and decreased Bcl-xL expression in Huh7 cells. SN-38 decreased p53 expression and increased P-gp expression after 120 h, resulting in inhibition of apoptosis. This inhibition was reversed by the addition of verapamil to the culture medium during 120 h incubation. SN-38-induced P-gp expression was additionally enhanced by p53 decoy oligodeoxynucleotide. The changes in P-gp expression were directly moderated by p53 gene downregulation, suggesting that it plays a role in the mechanism of drug resistance. These results suggest that the accumulation of irinotecan in HCC leads to the development of drug resistance.

Key words SN-38; p53; apoptosis; P-glycoprotein; hepatic cancer

Hepatocellular carcinoma (HCC) is one of the most common malignancies and its incidence appears to be increasing worldwide.1,2) The curative treatment is surgical resection, liver transplantation, and percutaneous ablation, but these are indicated in only 30% of patients.3) Furthermore, advanced tumors frequently recur early even after complete surgical resection. Therefore, many nonsurgical techniques have been developed and used for the treatment of inoperable HCC, although with limited efficacy.4) No anticancer agents have reproducibly shown high response rates.

Irinotecan is a derivative of camptothecin which inhibits topoisomerase (Topo-I) through the formation of stable Topo-I–DNA complexes5) and has been used as a chemotherapeutic agent for the treatment of several malignant or solid tumors.6) Irinotecan is converted by carboxylesterase to its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), in the liver. SN-38 is at least 1000-fold more cytotoxic than the parent compound and shows anticancer effect activity against a wide range of tumors.5–8) Although recent studies have suggested that SN-38 induces tumor regression through the apoptosis of several types of cancer cells,6,9) the detailed mechanisms of its anticancer effects and the development of drug resistance in HCC have not been clarified.

Recently, several investigators have suggested that apoptosis is an important mechanism in chemotherapy-induced tumor cell death. Apoptosis induced by irinotecan or SN-38 was demonstrated in colon carcinoma cells through poly(ADP-ribose) polymerase cleavage.9) SN-38 also arrests the cell cycle in G2 and induces apoptosis of the Bcl-2 family and p53 protein in testicular cancer.10) The p53 tumor-suppressor gene regulates the cell cycle and cell growth.11) Recent studies have demonstrated that p53 gene transfection into HCC cells increases chemosensitivity to specific anticancer drugs.12–14) Thus, p53 gene activation is currently considered to be a key target of anticancer drugs.

In addition, multidrug resistance in human cancers is a major obstacle to long-term, sustained patient responses to chemotherapy. Several mechanisms for the development of chemoresistance by cancer cells have been suggested, including inhibition of apoptosis, overexpression of the multidrug resistance (MDR) gene, and activation of antiapoptotic transcription factors.15,16) Acquired and intrinsic drug resistance to irinotecan and SN-38 has frequently been correlated with the presence of drug transporter proteins.17) P-Glycoprotein (P-gp) is a member of the ATP-binding cassette family of transporters encoded by the MDR-1 gene. P-gp functions as an active proton efflux pump for various anticancer agents.18,19) Overexpression of P-gp is associated with a poor response to chemotherapy. HCC patients are known to have elevated levels of the MDR-1 gene20,21) and their drug resistance may be related to this. The expression of P-gp in cancer cells may be an important determinant of the efficacy of anticancer agents. Although there is good evidence for a correlation between MDR-1/P-gp expression and chemoresistance in HCC, the molecular mechanisms of drug resistance to chemotherapy with irinotecan and SN-38 have not been fully elucidated. It is therefore necessary to investigate changes in the expression of P-gp with the administration of SN-38 and irinotecan. Chu et al. reported that P-gp is involved in the active efflux of SN-38 and irinotecan.22) Furthermore, several studies have shown that MDR-1/P-gp expression is rapidly upregulated in the presence of SN-38 and irinotecan.23–25) The aim of the present study was to define the correlation between anticancer activity of SN-38 and irinotecan and the in-
duction of drug resistance using a short-term culture model. To elucidate the processes and mechanisms of SN-38-induced cancer cell apoptosis and resistance through P-gp expression, in this study we examined the effects of continuous administration of SN-38 on HCC cell apoptosis and the contribution of P-gp to drug resistance in the HCC cell line Huh7.

MATERIALS AND METHODS

Drugs and Antibodies SN-38 was obtained from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). The drug was dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C until use. Verapamil (Wako Pure Chemicals, Osaka, Japan) as an agent to reverse the activity of P-gp was dissolved in RPMI medium and stored at −20 °C until use. Antibodies used in the present study were: anti-p53 polyclonal antibody (Ab) (Chemicon International Inc., Temecula, CA, U.S.A.), anti-P-gp Ab, anti-Bcl-xL polyclonal Ab, anti-Bax polyclonal Ab, and anti-caspase-9 polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.).

Cell Lines and Cell Cultures The HCC cell line Huh7 was obtained from the Riken Gene Bank (Tsukuba, Japan). The cells were cultured with RPMI-1640 medium (Invitrogen, Grand Island, NY, U.S.A.) containing 10% fetal bovine serum (Gibco BRL, Grand Island, NY, U.S.A.) with penicillin 100 units/ml and streptomycin 100 mg/ml at 37 °C under an atmosphere of 5% CO2 and 95% O2. The cells were seeded at 5.0×10⁴ cells in culture dishes; they were incubated for 24 to 120 h. They were also preincubated with 3 mM or 5 mM ODN. Thereafter, SN-38 was added to each sample and cultured for 120 h. Preincubation with 3 mM or 5 mM ODN. Thereafter, SN-38 was added to each sample and cultured for 120 h. Total RNA (2 μg) was reverse-transcribed with a RETROscript kit (Ambion Inc., Austin, TX, U.S.A.) in a final volume of 20 μl at 42 °C for 1 h.

Real-Time Polymerase Chain Reaction cDNA was synthesized using the reverse-transcription (RT) reaction as described above, and quantitative analysis was performed with a 3520S Light Cycler (Roche Diagnostics, Basel, Switzerland) as described previously with slight modification. Briefly, the polymerase chain reaction (PCR) was performed in 20 μl of total reaction volume containing 2 μl cDNA, 10 pmol of specific primers for MDR-1 or GAPDH, 3 mM MgCl₂, and 2 μl SYBER Green (Roche Diagnostics), according to the manufacturer’s protocol using LightCycler FastStart DNA Master SYBER Green Kit (Roche Diagnostics). The cycling protocol for MDR-1 consisted of one cycle of 10 min at 95 °C, followed by 40 cycles of denaturation for 15 s at 95 °C, annealing for 15 s at 62 °C, and extension for 10 s at 72 °C. The primers for MDR-1 were 5’TGGCCCGGCCCTCATC-3’ (sense) and antisense; 5’TTCATCCAATTCAGATCCAGAGCTGAT-3’ (antisense). The cycling protocol for GAPDH consisted of one cycle of 10 min at 95 °C, followed by 40 cycles of denaturation for 15 s at 95 °C, annealing for 10 s at 60 °C, and extension for 10 s at 72 °C. The primers for GAPDH were 5’GACAATTTTGATATCCGTGGGA-3’ (sense) and 5’TACCAGGAATGAGGTTGAC-3’ (antisense).

Western Blot Analysis The nuclear proteins in the cells were prepared using a commercial kit (NE-PER nuclear and cytoplasmic extraction kit, Pierce Biotechnology, Rockford, IL, U.S.A.), and whole proteins were also extracted with cell lysis buffer (50 mM Tris–HCl, 1 mM EGTA, 0.001% leupeptin, pH 7.4). The protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, U.S.A.). After boiling at 100 °C for 2 min, equivalent amounts of proteins (25 μg) were resolved upon 10% SDS-polyacrylamide gel electrophoresis. Thereafter, proteins were transferred onto Hybond-ECL membranes (Amersham Bioscience, Buckinghamshire, U.K.), for 60 min. The membranes were blocked with 2% skim milk for anti-P-gp Ab and anti-α-Tubulin Ab, 5% skim milk for anti-p53 Ab, and anti-Bax Ab and caspase-9 Ab and 2% BSA for anti-Bcl-xL Ab with Tris-buffered saline (0.1 M Tris–HCl, pH 7.5, 1.5 M NaCl) containing 0.05% Tween 20 (TTBS) overnight at 4 °C. The blots were probed with specific primary Abs in TTBS diluted to 1 : 200 for 2 h at room temperature. After washing three times for 10 min each with TTBS, the blots were then conjugated with horseradish peroxidase-conjugated secondary Ab against rabbit or mouse IgG at a 1 : 2000 dilution for 1 h at room temperature. Reactive proteins were detected in the chemiluminescence assay (ECL plus kit, Amersham Bioscience). The intensity of the detected bands was analyzed using gel-blotting macros in NIH image 1.65 software (Ver. 1.65, National Institutes of Health, Bethesda, MD, U.S.A.).

Detection of Induced P-gp by Immunofluorescence Microscopy The immunofluorescence microscopy assay was used to characterize P-gp functional activity in the cells treated with SN-38 25 ng/ml for 120 h. P-gp underwent conformational transitions that are detected by UIC2,25,30 a functional anti-P-gp monoclonal Ab (Chemicon International Inc.). Huh7 cells were cultured on microchamber slides and allowed to adhere for 24 to 120 h. They were also preincubated...
bated with 5 μM or 10 μM verapamil for 1 h, followed by the addition of SN-38. Thereafter, the slides were fixed in cold acetone for 15 min. The first antibody of the mouse IgG2a as a control and MDR-1 mAb were applied to the slide and incubated for 30 min at 37 °C. All subsequent procedures were performed using a MDR-1 shift assay kit as instructed by the manufacturer (Chemicon International Inc.). The UIC2-positive cells were detected with fluorescence microscopy using a Carl Zeiss Axiophot microscope (Hitchscifel Optical Institute, Inc., St. Louis, MO, U.S.A.).

Statistical Analysis Data are expressed as mean±S.E.M. Differences between groups were analyzed using two-way ANOVA, followed by comparison with the Mann–Whitney U-test or Fisher’s PLSD test. A p value of less than 0.05 was considered to represent a statistically significant difference.

RESULTS

Effects of Accumulated SN-38-Induced Apoptosis in Huh7 Cells When the effects of apoptosis in SN-38-treated Huh7 cells were analyzed using TUNEL staining (Fig. 1), there was no significant difference in the number of apoptotic cells in the control cells at 24 h, 72 h, or 120 h (5.73±0.71%, 6.74±1.43%, and 5.28±2.48%, respectively). After the incubation of Huh7 cells with SN-38 12.5 ng/ml, a continuous increase in apoptosis was seen compared with the control cells at 120 h (15.17±1.44% at 24 h, 19.35±2.29% at 72 h, and 33.03±4.65% at 120 h, p<0.01). The SN-38 doses of 25 and 50 ng/ml significantly and dose dependently increased the number of apoptotic cells from 24 h (23.68±2.01%, and 28.85±1.77%, respectively, p<0.01) to 72 h (28.85±1.77%, and 34.15±2.43%, respectively, p<0.01) compared with the number in the control cells. However, after treatment with SN-38 25 ng/ml (22.53±2.69%) or 50 ng/ml (21.03±2.81%, p<0.05) for 120 h, apoptotic cells were decreased compared with the number in cells treated with the same dose of SN-38 for 72 h (Fig. 1).

Effects of SN-38 on p53 Protein Expression in the Nuclei and Apoptosis-Related Proteins in the Cytoplasm of Huh7 Cells We next examined the effects of SN-38 on p53 protein expression in the nucleus and apoptosis-related proteins in the cytoplasm of Huh7 cells using Western blot analysis. After treatment with SN-38 25 ng/ml for 24 h, p53 protein expression in the nuclei of these cells increased significantly compared with that in control cells. In contrast, after treatment with SN-38 for 120 h, p53 protein expression in Huh7 cells decreased significantly compared with that in control cells (Fig. 2). The effects of SN-38 on apoptosis-related protein expression are shown in Fig. 2. After treatment with SN-38 for 24 h, Bax and caspase-9 protein expression in these cells increased compared with that in control cells, while antiapoptotic protein Bcl-xL expression decreased significantly compared with that in control cells. On the other hand, Bax and caspase-9 protein expression in Huh7 cells after treatment with SN-38 25 ng/ml for 120 h decreased significantly compared with that after 24 h treatment. Bcl-xL protein expression in Huh7 cells after treatment with SN-38 for 120 h increased significantly compared with that after 24 h treatment.

Fig. 1. Effects of SN-38 Accumulation on Apoptosis in Huh7 Cells
Huh7 cells (2×10^4 cells) were cultured in a microchamber for 24, 72, or 120 h in the presence of SN-38 12.5, 25, or 50 ng/ml, respectively. TUNEL-positive cells, considered to be apoptotic cells, were counted and are shown as the percentage of apoptotic cells in total cells. Bars indicate mean±S.E.M. Data are representative of 12 independent experiments. Details are described in Materials and Methods. * p<0.01 vs. control cells, † p<0.05 vs. 72 h of incubation with SN-38 50 ng/ml.

Fig. 2. Effects of SN-38 Accumulation on p53 Protein Expression in the Nuclei and Apoptosis-Related Proteins in the Cytoplasm of Huh7 Cells p53 protein expression in the nuclear fraction and apoptosis-related protein expression in the cytoplasm of Huh7 cells treated with SN-38 25 ng/ml for 24, 72, and 120 h were determined using Western blot analysis. α-Tubulin was used as the loading control. Upper panel shows the representative bands of p53, Bcl-xL, Bax, and caspase-9. Lower panel shows quantitative results of proteins detected in densitometric analysis. All data are expressed as mean±S.E.M. and are representative of six independent experiments. Details are described in Materials and Methods.
The effects of SN-38 on P-gp expression are shown in Fig. 3. (A) Cells were cultured with SN-38 for 24, 72, and 120 h, and then MDR-1 mRNA expression was analyzed using semiquantitative real-time PCR. The expression of MDR-1 mRNA relative to GAPDH mRNA is shown as mean ± S.E.M. Data are representative of six independent experiments. (B) Western blot analysis of P-gp expression in Huh 7 cells treated with SN-38 25 ng/ml for 24, 72, and 120 h. α-Tubulin was used as the loading control. Data are expressed as mean ± S.E.M. and are representative of eight independent experiments. (C) P-gp induced using UIC2 monoclonal Ab to recognize an extracellular conformational epitope as detected by fluorescence microscopy. Data are representative of three independent experiments. Original magnification ×200. Details are described in Materials and Methods.

In the present study, the human HCC cell line Huh7 used was derived from Japanese patients with HCC and produced high levels of alpha-fetoprotein. We found that SN-38 increased apoptotic cell death associated with changes in levels of apoptosis-related proteins after 24 h incubation of Huh7 cells with SN-38 (Fig. 1). We thus confirmed that SN-38 induced apoptosis and increase in Bcl-xL expression were reversed in Huh7 cells preincubated with verapamil 10 μM, followed by the addition of SN-38 25 ng/ml for 120 h (Fig. 4C).

Effects of p53 Decoy ODN on SN-38-Induced P-gp Expression in Huh7 Cells To determine whether increased P-gp expression contributes to the decreased p53 expression after SN-38 accumulation in Huh7 cells, p53 decoy ODN to inhibit p53 activation was used in the experimental system. The cells were precultured with 3 μM or 5 μM p53 decoy ODN, followed by the addition of SN-38 for 120 h. SN-38-induced P-gp expression was significantly enhanced in p53 decoy ODN-precultured Huh7 cells (Fig. 5A). Treatment with p53 decoy ODN alone was confirmed to have no effect on P-gp expression and apoptosis of Huh7 cells in this experimental system (Fig. 5A). The addition of 5 μM p53 decoy ODN to the culture medium resulted in greater activation of P-gp than that in Huh7 cells treated with SN-38 25 ng/ml for 120 h (Fig. 5B).

p53 decoy ODN-precultured Huh7 cells also exhibited a significant decrease in the number of TUNEL-positive cells compared with SN-38-treated cells (Fig. 5C).

DISCUSSION

In the present study, the human HCC cell line Huh7 used was derived from Japanese patients with HCC and produced high levels of alpha-fetoprotein. We found that SN-38 increased apoptotic cell death associated with changes in levels of apoptosis-related proteins after 24 h incubation of Huh7 cells with SN-38 (Fig. 1). We thus confirmed that SN-38 induces apoptosis in these cells, and the results suggest that important apoptotic mechanisms are related to the anticancer effects of irinotecan without the inhibition of Topo-I. Apoptosis is modulated through a series of protooncogenes and tumor-suppressor genes. Upon activation by DNA damage-induced or oncogene-induced signaling pathways, p53 promotes the expression of several genes that are involved in apoptosis, including those encoding death receptors and proapoptotic members of the Bcl-2 family. Hayward et al. demonstrated that SN-38 induced the accumulation of p53 in HCT116-wt colorectal cancer cells. Wang and El-Deiry reported that irinotecan increases p53 transcriptional activity in vivo. We showed that p53 expression in SN-38-treated Huh7 cells was significantly greater than that in controls at 24 h, suggesting that SN-38-induced p53 expression may be related to transcriptional mechanisms.
Furthermore, SN-38 increased the apoptosis of Huh7 cells after 24 h incubation, associated with increases in levels of the apoptosis-accelerating proteins Bax and caspase-9, and decreased the levels of the apoptosis-inhibitory protein Bcl-xL (Fig. 2). These results suggest that SN-38-induced apoptosis of Huh7 cells mediates the increased p53 expression and involves the mitochondrial pathway through changes in Bax and Bcl-xL expression levels.

On the other hand, alterations in the regulation of the apoptotic process potentially lead to the failure of therapy. Clinical drug resistance to irinotecan is frequently observed. Nicolantonio et al. reported that short-term (6-d) culture of cancer cells with anticancer agents provides a suitable model for studying resistance mechanisms.26) We also observed that the incubation of Huh7 cells with SN-38 for 120 h resulted in a significant decrease in the number of apoptotic cells compared with 24 h incubation (Fig. 1). Thus, SN-38-induced apoptosis gradually decreased over time. This finding indicates that, at least in part, the reduction of apoptosis by SN-38 accumulated in the cells for 120 h might be related to drug resistance to irinotecan.

Furthermore, SN-38 increased the apoptosis of Huh7 cells after 24 h incubation, associated with increases in levels of the apoptosis-accelerating proteins Bax and caspase-9, and decreased the levels of the apoptosis-inhibitory protein Bcl-xL (Fig. 2). These results suggest that SN-38-induced apoptosis of Huh7 cells mediates the increased p53 expression and involves the mitochondrial pathway through changes in Bax and Bcl-xL expression levels.

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resistance against cancer chemotherapy. In addition, p53 expression in SN-38-treated cells gradually decreased in a time-dependent manner (Fig. 2). Interestingly, the expression of Bax and caspase-9 gradually decreased and that of Bcl-xL gradually increased in SN-38-treated cells over time and was correlated with the change in the number apoptotic cells (Fig. 2). The decrease in p53 expression was also associated with the changes in Bax and Bcl-xL expression. Bax is a major mediator of p53-dependent apoptosis, and a proapoptotic heterodimeric partner of Bcl-xL.\(^{38}\) These data suggest that the decreased apoptosis with the administration of SN-38 is related to p53-induced changes in Bax and Bcl-xL expression via the mitochondrial pathway.

Several mechanisms of drug resistance to irinotecan have been identified in preclinical models. These include increased efflux of irinotecan out of cells, which may involve P-gp and multidrug-resistance proteins.\(^{39,40}\) Few data are available on the relative contribution and frequency of these events in patients whose cancer progresses while being treated with irinotecan. P-gp acts as an efflux pump for many drugs.\(^{41}\) Several investigators reported that P-gp-related drug resistance was observed in P-gp-knockout cells or P-gp-overexpressing cells.\(^{42,43}\) Overexpression of P-gp is involved in the active efflux of SN-38 and irinotecan and indicates a reduction in the intracellular accumulation of SN-38.\(^{39}\) However, to the best of our knowledge, there was no previous study on the process by which P-gp expression is induced after exposure to SN-38. In the present study, we found that continuous administration of SN-38 induced apoptosis in association with increases in MDR-1 mRNA levels and P-gp activation in a time-dependent manner (Fig. 3). These data suggest that \textit{de novo} acquired drug resistance to irinotecan may be related to SN-38-induced increases in P-gp expression after short-term culture with the drug. Verapamil appears to be the only inhibitor that is uniquely associated with P-gp and forms a good substrate for P-gp. To elucidate the role of P-gp in the decreases in SN-38-induced apoptosis, we investigated the effects of verapamil on changes in apoptosis after SN-38 treatment. Inhibition of SN-38-induced apoptosis through the mitochondrial pathway was reversed and/or increased by the addition of verapamil to HuH7 cells at 120 h (Fig. 4). Therefore, one mechanism of SN-38 resistance may be related to the transporter of P-gp. Furthermore, it is possible that as intracellular levels of P-gp increase, SN-38 is pumped out of the cells, thereby decreasing intracellular SN-38 levels. On the other hand, the possible involvement of MRP family transporters in the development of drug resistance cannot be ruled out. The roles of other transporters in decreasing the apoptosis rate after SN-38 saturation should be clarified in further investigations.

To clarify the molecular events involved in the interaction between P-gp and p53, we utilized p53 53c3 decay ODN to block the function of p53 in SN-38-saturated Huh7 cells. The P-gp activation induced by SN-38 saturation was enhanced by p53 53c3 decay ODN (Fig. 5). These changes in P-gp were directly modulated by p53 gene downregulation, suggesting that it plays a role in the mechanism of drug resistance. Zhan \textit{et al.} reported that increased p53 expression leads to decreased P-gp phosphorylation.\(^{45}\) Our data may suggest that p53 not only decreases P-gp phosphorylation but also decreases P-gp expression due to the effects of p53 on P-gp. However, the detailed mechanism of the p53-induced decrease in P-gp protein expression was unclear in this study.

In conclusion, our findings in this study show that the expression of P-gp induced by short-term culture of HCC with SN-38 is associated with the inhibition of apoptosis \textit{via} p53 mitochondrial pathway and thus p53 gene downregulation affects the chemosensitivity to irinotecan. We therefore suggest that the inhibition of apoptosis in HCC is due to acquired resistance with SN-38 accumulation.

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